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Abstract:
Vaccinations against the encephalitic alphaviruses (western, eastern, and Venezuelan equine encephalitis) are of significant interest to agricultural and biological defense communities alike. Although vaccines licensed for veterinary applications are utilized in the Western Hemisphere and attenuated or inactivated viruses have been used under Investigational New Drug status to protect at-risk personnel, there are currently no licensed vaccines for use in humans. Here, we will discuss the need for a trivalent vaccine that protects humans against all three viruses, recent progress towards such a vaccine, and a strategy to continue development towards Food and Drug Administration licensure.

Introduction:
Western (WEEV), eastern (EEEV), and Venezuelan (VEEV) Equine Encephalitis Viruses represent three encephalitic alphaviruses for which vaccines are needed. The requirement for these vaccines is due in large part to the severe disease caused by each of these viruses and their potential for use as biological weapons, which has resulted in their identification as Category B pathogens by the National Institute of Allergy and Infectious Diseases. Veterinary vaccination programs are currently utilized to protect equine populations in a number of countries in the Western Hemisphere, but human vaccines are limited to those that have achieved Investigational New Drug (IND) status and are used only to protect laboratory workers handling the viruses.[1] Additional emphasis is needed from the science and technology base through advanced development in order to develop a Food and Drug Administration (FDA)-licensed vaccine against all three of these pathogens.

The alphaviruses are members of the *Togaviridae* family of enveloped viruses. Prototypical alphaviruses utilize a single-stranded, positive-sense RNA genome that encodes four nonstructural and five structural proteins.[2] These viruses are zoonotic pathogens, maintained by mosquitoes and birds (WEEV and EEEV) or mammals (VEEV) in an enzootic life cycle.[3] Depending on climate conditions, mosquito species, and/or virus strain, they can also be transmitted to horses and humans via spillover of enzootic transmission or in an equine-amplified, epizootic cycle causing severe encephalitis in some cases.[3]

The geographic distribution and genomic diversity varies among these three viruses. WEEV can be isolated from birds and mosquitoes, mainly *Culex tarsalis*, across the western parts of the Americas from as far north as southern Canada and south to Argentina and Brazil.[4-5] WEEV is a natural recombinant virus, containing envelope proteins from a Sindbis virus-like ancestor and non-structural and capsid proteins from an EEEV-like ancestor.[6] Comparisons of isolates from North and South America reveal several antigenic subtypes and viral lineages,[7-8] with variation in virulence in rodent models.[9-11]

In North America, EEEV circulates between birds and *Culex melanura* or other mosquitoes, but transmission to humans or horses can occur via *Aedes*, *Coquillettidia*, or *Culex* mosquito species that feed on both birds and mammals.[3] Isolates can be described as North or South American antigenic varieties based on hemagglutinin inhibition assays, and four antigenic subtypes are delineated using neutralization assays.[12] North American isolates are also noted as belonging to the Group I lineage and cause the majority of documented human illnesses. South American isolates in Groups IIA, IIB, and III are responsible for equine outbreaks in Central and South America and recently were reclassified as the distinct species *Madariaga virus*.[12-13] Until recently, it was thought that Central and South American EEEV strains were not a major concern in terms of human health. However, recent analyses of EEEV cases in a 2010 Panamanian outbreak may change that view and warrant further investigation of these isolates.[14]

VEEV is one of several species in the VEE antigenic complex of alphaviruses. The life cycle of most of these viruses involves enzootic transmission between rodents and mosquitoes, with outbreaks of epizootic disease being associated with the virus subtype and specific mosquito vectors. It is mainly restricted to Central and South America and is the most diverse of the three viruses with six different subtypes (I-VI) that can be distinguished by serology. Furthermore, there are five different variants within subtype I (AB, C, D, E, and F). The epizootic variants, IAB and IC, are thought to evolve periodically from ID ancestors and cause the majority of epizootic disease in humans and horses.[15-16] The ID, IE, and IIIA strains are also capable of causing febrile disease in humans, but these and the remaining subtypes are primarily associated with enzootic cycles.[3] The diversity of VEEV subtypes creates an additional challenge for vaccine design which will be discussed further below.

Although WEEV, EEEV, and VEEV are all capable of causing lethal encephalitis in humans and horses, the incidences and courses of disease do vary substantially among them. WEEV is somewhat rare, with fewer than 700 cases reported in the United States since the 1960's and no human infections detected since 1988.[17] The rate of asymptomatic versus symptomatic WEEV infection varies widely with age, with children below the age of one being most susceptible to severe disease and the overall mortality rate being estimated at about four percent.[1] Similarly, there are typically only a few cases of EEEV in the United States each year, although many more undetected, asymptomatic infections may occur. However, clinical cases of EEEV infection have a much higher mortality rate estimated at 30-70 percent and survivors often experience severe, permanent neurological sequelae.[3,18] VEEV appears to have the greatest potential for epizootic disease although mortality rates are typically only around one percent and mainly involve children.[18] Flu-like symptoms are typical of an infection, with many patients recovering in three to five days. One of the largest VEEV outbreaks occurred in Columbia in 1995 and affected approximately 75,000 individuals. Among this population, 3,000 people developed neurological signs and symptoms and the outbreak resulted in about 300 deaths.[19] It is important to note that there is substantial evidence that VEEV is also highly infectious via an aerosol route of exposure. This is highlighted by over 150 documented cases of accidental laboratory exposures in the literature, some of which are presumed to have occurred via the aerosol route.[20] This has in part resulted in an increased emphasis on medical countermeasure development for VEEV, EEEV, and WEEV from a biodefense perspective.

Need for a Trivalent Western, Eastern, and Venezuelan Equine Encephalitis (WEVEE) Vaccine

There are currently no FDA-approved vaccines or antiviral medical countermeasures available to protect against or treat infection by WEEV, EEEV, or VEEV. There are two major factors to be considered when discussing the need for a trivalent WEVEE vaccine; the need from a biodefense perspective and the costs

associated with treating severe illness caused by WEEV, EEEV, or VEEV. These encephalitic alphaviruses were developed as agents of biological warfare prior to the Biological Weapons Convention Treaty.[21] These viruses were pursued for biological warfare applications largely due to the ease of producing high quantities of aerosolized viruses, and their highly infectious nature via aerosol exposure. Furthermore, the cost associated with supportive treatment of endemic cases can range from $21,000 for mild infections up to $3 million for severe encephalitis in the case of WEEV.[22] An effective vaccine would greatly limit the threat posed by these viruses; as a result, the Defense Threat Reduction Agency-Joint Science and Technology Office and Joint Program Executive Office-Medical Countermeasure Systems have formed a translational team to facilitate advancement of candidate vaccines from the technology base through advanced development towards FDA licensure.

Vaccines against encephalitic alphaviruses are of also of significant interest to the agricultural community as well. In the 1960s and 1970s, VEEV outbreaks in Columbia, Central America, Mexico, and the United States, warranted vaccination of over 2 million equids with the live vaccine strain TC-83, which was attenuated by 83 passages through cultured guinea pig heart cells. Although TC-83 was quite successful in limiting the spread of the 1971 Texas VEE epizootic, two key issues were noted. In early studies, signs of disease were observed in five out of ten horses vaccinated with TC-83 and viremia was observed in eight of those animals, suggesting that the attenuated virus could be transmitted back to mosquitoes.[23-24] This also further highlighted the second issue, the potential for reversion back to virulence given that attenuation relies on only two point mutations.[25] In an attempt to mitigate the concern of virulence reversion, a new live-attenuated virus vaccine (V3526) was derived by site-directed mutagenesis of the Trinidad donkey strain of VEEV subtype 1A/B. Development of V3526 was continued to the point of testing in a horse model of infection and demonstrated promising results in terms of safety and protection.[26] An inactivated version of TC-83 (C84) is also available in North America, but is typically only recommended in high-risk areas. As a result, TC-83 continues to be produced in Colombia and Mexico for use in equids. Vaccination against WEEV and EEEV is currently recommended for horses living in, or traveling to, North America due to exposure risks and the high mortality associated with these viruses in equine hosts.[27] A bivalent preparation is typically utilized, consisting of formalin-inactivated, adjuvanted whole viruses.

TC-83 is also currently used for laboratory workers and other at risk personnel under IND status. However, there are substantial issues with both the immunogenicity and safety of this vaccine candidate. Out of more than 800 individuals who received the vaccine between 1976 and 1990, nearly 20 percent did not respond as measured by neutralizing antibody titers.[28] Furthermore, over 20 percent of individuals experienced reactogenicity issues including fever, muscle aches, and headaches.[28] Boosts with C84 are often required for non-responders to TC-83. Formalin-inactivated EEEV and WEEV vaccines have been used under IND status to vaccinate at risk laboratory workers as well. It is important to note that multiple studies have now shown that there is potential for immune interference when these vaccines are utilized in an attempt to protect against all three species; immune interference has been observed when vaccinations are given both sequentially and simultaneously.[29-30] Therefore, the combination of safety, immunogenicity, and immune interference issues have warranted further investments in a trivalent WEVEE vaccine to fulfill the requirements of the biodefense community.

Recent progress towards WEVEE vaccines

The research community has continued to pursue safer and more effective vaccines against the encephalitic alphaviruses, with many groups emphasizing VEEV due to its potential for epizootic outbreaks. Efforts have been made to develop next-generation vaccines via a number of platforms; live-attenuated virus chimeras and targeted mutants, viral replicons (VRP), virus-like particles (VLP), DNA-

based approaches, and an array of different inactivation techniques for whole viruses.[31-34] The number of candidate vaccines that have been developed for individual virus species is much larger than the number that have been tested for potential utility as trivalent vaccines. Table 1 highlights the progress towards a trivalent vaccine against WEEV, EEEV, and VEEV, which has largely focused on inactivated viruses, VRPs, VLPs, and DNA-based vaccines.

Various formulations of inactivated virus have been tested in recent years to include different mechanisms of inactivation and routes of administration. These approaches have typically involved a mixture of the three species, inactivated by formalin, 1,5-iodonapthyl-azide, or gamma irradiation. The impact of the route of administration has also been investigated including intramuscular, subcutaneous, and intranasal delivery of the inactivated virus. Immunogenicity and protective efficacy have been shown to vary somewhat as functions of the inactivation strategy and route of administration.[35-36] [(unpublished data)] However, a formulation that elicits an optimal immune response has not been achieved, resulting in decreased emphasis on inactivated candidates.

The VRP platform being pursued is based on an attenuated strain of VEEV into which the genes coding for various structural proteins can be inserted.[37] In the case of the WEVEE vaccine candidate, this involves expression of the alphavirus glycoproteins with deletions in the PE2 furin cleavage site. Protective efficacy has been observed in both mouse and non-human primate (NHP) models of infection with a trivalent WEVEE VRP formulation[unpublished data] as well as VLP and DNA-based vaccines, providing the proof-of-concept that a trivalent vaccine may be feasible.

A variety of VLPs have been pursued for vaccination against alphaviruses, however, the most mature has stemmed from initial work with Chikungunya virus. Expression of the virus structural proteins resulted in a VLP that resembled the structure of live alphaviruses.[38] This VLP was shown to elicit a robust antibody response and protection against Chikungunya virus.[38] These methods are now being applied to WEEV, EEEV, and VEEV, where monovalent preparations were shown to be protective against all three viruses in both mice and non-human primates with no detection of viremia.[unpublished data] Trivalent preparations of the three VLPs are currently being evaluated for immunogenicity and protective efficacy in mouse and NHP models of infection.

The DNA-based vaccine consists of a plasmid with an insert encoding the E1 and E2 viral proteins. The DNA is delivered by intramuscular or intradermal electroporation, which facilitates uptake of the DNA by host cells. Once inside the cells, the viral genes are transcribed and translated by host cell machinery to yield E1 and E2 proteins.[39] A robust antibody response is elicited, with neutralizing antibodies being suggested as a correlate of immunity. Protection against aerosol challenge with all three viruses has been observed in mice and nonhuman primates, both in terms of survival and the prevention of viremia.[39] Importantly, there is no reduction in immunogenicity or protective efficacy when the vaccines are given as a trivalent formulation, as opposed to monovalent formulations. In terms of vaccines to be utilized for protection of the Warfighter, the administration of the DNA-based vaccines by electroporation poses a logistical challenge in its present format in that it requires an electrical device for effective delivery. However, next generation devices suitable for field deployment are under development and are likely to greatly reduce the logistical issues associated with these first generation clinical devices. Nevertheless, the success of the DNA vaccines as a trivalent WEVEE vaccine suggests that studies to identify simpler delivery platforms may be warranted as well.

Path from experimental vaccines to licensure

As noted in the previous section, multiple vaccines have shown promising efficacy against WEEV, EEEV, and VEEV in established animal models. However, advancing these candidates to licensure will require continued emphasis from both the science and technology base and the advanced development community in the near future. This section will begin to touch on a strategy that may be used to ultimately deliver a safe and effective vaccine against WEEV, EEEV, and VEEV to the Warfighter.

Regarding the protection of Warfighters against the encephalitic alphaviruses, efficacy against an aerosol exposure is the primary goal given the potential biological threat. However, with no cases of aerosol occurring in nature, and a limited number of documented laboratory exposures, approval for an aerosol indication would likely occur via the FDA Animal Rule. That being the case, the development of well-characterized models of aerosol exposure to WEEV, EEEV, and VEEV is paramount to advancing vaccine candidates towards licensure. Equine species are natural hosts in terms of the epizootic disease, but are limited in their utility as experimental models of encephalitic alphaviruses due to their size. Therefore, substantial development of both small and large animal models of aerosolized WEEV, EEEV, and VEEV infection has been pursued.

Animal Model Development

Mouse models of infection have been pursued extensively for all three viruses and offer the benefits of well-characterized immunological reagents and congenic mutant strains that can help to tease out mechanisms of pathogenesis and immunological protection. Peripheral VEEV infection of mice is lymphotropic and entrance into the central nervous system (CNS) occurs only after viremia seeds infection of the olfactory neurons.[40] Aerosol infections of mice by VEEV have demonstrated a tropism for the olfactory neuroepithelium, resulting in more rapid neuroinvasion relative to a subcutaneous exposure.[41-42] VEEV is able to infect a range of neuronal cell types and lesions are observed in both neural and non-neural tissues. Utilization of an array of attenuated virus strains and immunodeficient mouse strains has elucidated both viral and host factors that play key roles in VEEV pathogenesis. EEEV infection of mice also results in severe neuroinvasion, but is more age-dependent than VEEV. Unlike VEEV, EEEV is not lymphotropic and its route of CNS invasion via the bloodstream after subcutaneous exposure remains poorly characterized. In contrast, exposure via the aerosol route involves neuroinvasion via the olfactory system much like VEEV.[43] Otherwise, many of the general pathological findings appear to be similar to what is often observed in clinical cases. WEEV neuroinvasion and neurovirulence varies widely by strain, and like EEEV its mechanism of entry into the CNS is not well understood. Aerosol models of WEEV exposure in mice have also been documented with neuronal lesions, but not with the same amount of detail regarding its pathophysiology relative to the other alphaviruses. In all, the mouse models of aerosol exposure of the encephalitic alphaviruses have been characterized rather thoroughly and have provided useful tools in the initial assessment and down-selection of vaccine candidates.

Licensure of a WEVEE vaccine by the Animal Rule will likely require a well-characterized large animal model in which pivotal animal studies can be conducted. In this light, the cynomolgus macaque (*Macaca fascicularis*) would be a logical choice to pursue given 1) the observed pathophysiology, 2) the relation to human disease, and 3) the amount of work in this species described in the literature. Similar to the human condition for VEEV, most infected cynomolgus macaques develop a transient fever and viremia, occasionally with neurological signs, but eventually recover from the infection.[44] While many of the past studies were limited in terms of the technologies that were available to analyze the tissues, the general disease progression is similar to that observed in humans. Additional macaque infections via the

aerosol route with more recent clinical isolates will be useful to provide detailed pathophysiological findings and comparisons to past experiments involving subcutaneous exposures.

Relative to VEEV, there have been far fewer studies involving experimental infections of NHPs with EEEV. However, the results of these experiment suggest that cynomolgus macaques may also be a suitable model for EEEV. Infection by EEEV was lethal via aerosol challenge with large doses, with eight out of 12 animals developing fever and neurological signs. The animals also succumbed to infection within 5-9 days, which is consistent with the high mortality associated with human disease relative to the other alphaviruses.[45] Similarly, there have been a limited number of WEEV infections in the cynomolgus macaque, but these have also suggested utility for this non-human primate species as a model for WEEV. Macaques developed fever within 4-5 days as well as leukocytosis and hyperglycemia. This was accompanied by lesions in the brain with WEEV antigens being identified in microglial cells and neurons.[46] As is typical of WEEV infection in humans, the lethality rate was low, with one of six animals in each of the high and low dose groups succumbing to disease.

Infection of the cynomolgus macaque by WEEV, EEEV, or VEEV results in many similar pathological findings relative to human disease,[44] but a significant amount of work remains to be completed in order to thoroughly characterize the infectivity and lethality of the alphaviruses by the aerosol route. This will need to include detailed analyses of the physiological and immunological responses to the virus. However, the choice of virus strain will also be a critical aspect in further development of the cynomolgus macaque models of WEEV, EEEV, and VEEV infection.

Strain Selection

In selecting the strains of viruses to incorporate into animal model development, there are a few major criteria that must be considered. Isolation from human clinical cases is a first critical checkpoint for isolates to be considered. WEEV, EEEV, and VEEV can all readily be isolated from their respective vector and reservoir hosts, but not all isolates may be able to cause human disease. Thus, it is important to choose isolates that are known to be capable of causing the disease in humans. Passage history is another key concern for these viruses. Studies with VEEV have demonstrated that removal of selective pressures associated with a two-host life cycle result in accumulation of single nucleotide polymorphisms,[47-48] and cell culture passages often result in adaptation for heparin sulfate binding, which can result in artificial attenuation.[49] Isolates with low numbers of passages and well-characterized passage histories would be a substantial benefit to the animal models.

Relevance to currently circulating strains should also be considered, which would be supported by more extensive sampling and characterization of clinical isolates. VEEV presents an additional concern in that IAB strains were involved in offensive programs in the past, but IC strains are more relevant to recently circulating virus subtypes. The fact that there are multiple subtypes that could be a concern in terms of clinical illness poses a key challenge in terms of appropriate strain selection. That being said, there are multiple isolates for each virus that are quite suitable for inclusion in the development of the cynomolgus macaque model of infection.

Beyond the isolates used in the animal models, additional isolates may be of interest to test the breadth of protection conferred by a vaccine. It is important that a trivalent WEVEE vaccine protect against the most relevant subtypes and strains of each viral species; thus it will be essential to demonstrate cross-protection against a panel of isolates to determine the breadth of efficacy. EEEV is a fairly conserved species in terms of genomic diversity of strains with known human virulence. Until recently, it was believed that only Group I North American strains were a concern in terms of human disease. However,

recent analyses of a 2010 outbreak in Panama have challenged that paradigm.[50] Given that these strains caused severe disease in humans, further studies in animal models may be warranted in order to determine if and how these strains have evolved towards virulence. If these strains prove to be more virulent than other typical Central and South American strains, an assessment of immunological cross-reactivity may be essential to ensure sufficient protection of a licensed vaccine.

WEEV is even more conserved than EEEV, although it is poorly represented in public databases. WEEV has been suggested to be evolving towards virulence at a cost of infectivity of mosquito vectors,[51] but it is difficult to determine due to the paucity of genomic sequence data available, and variability within the animal models. Differential virulence has been observed among WEEV isolates, associated with changes in the E2 protein.[51] For both WEEV and EEEV, additional genomic sequencing and phylogenetic analyses of clinical and veterinary isolates, as well as comparative virulence studies in relevant animal models, would provide a solid rationale to define a panel of isolates against which immunological cross-reactivity should be measured.

As noted above, VEEV may present additional challenges due to the number of subtypes, which may necessitate further genomic characterization of clinical isolates and analyses of the virulence in defined animal models. The structural proteins, specifically E1 and E2, comprise the antigens that are being included in many of the experimental trivalent WEVEE vaccine constructs. Thus, the genomic variation among VEEV isolates in the E1 and E2 genes could be especially important in considering a panel of strains against which vaccine efficacy could be measured.

Preliminary analyses have shown that E1 and E2 have lower resolution for strain discrimination than the whole genome, with E2 having better resolution than E1. However, the envelope genes do capture the same higher level clades in the phylogeny as the full genome. The tanglegram in Figure 1 illustrates that 60 percent of splits from the whole genome were observed in trees built from just the gene for E1. The observed differences were not in the major branches of the phylogeny, but in the fine-scale arrangement of strains within clades. Similarly, Figure 2 illustrates the phylogenetic differences between E1 and E2. This also shows that major genome groupings are the same, and only the relationship among closely related strains within clades differs between E1 and E2. In particular, the strain relationships differ between E1 and E2 among isolates from a 2008-2010 outbreak of a VEEV IE subtype in Mexico, and a subset of IC and ID isolates from Venezuela, Columbia, and Peru (Figure 2). Further analyses have determined which single-nucleotide polymorphisms (SNPs) have allele variants that are non-randomly associated with given subtypes using chi-square p-values, and calculated separately for each subtype versus the others (i.e. type IC vs not IC). For nonsynonymous SNPs, there were 20 and 46, for E1 and E2 respectively, that were non-randomly associated with a subtype (Hall BG, manuscript in prep.). Together, these data suggest that focusing on gene variation in E1 and E2 would identify strains that would be of particular interest for heterologous challenge studies while also displaying the general diversity across major clades for VEEV. A greater understanding of the genomic diversity of these viruses and how that diversity may impact virulence will provide a sound rationale for the selection of a panel of WEEV, EEEV, and VEEV isolates against which immunological cross-reactivity would need to be analyzed.

Path to FDA Licensure

In seeking a vaccine that will protect against WEEV, EEEV, and VEEV via aerosol exposure, there are several key aspects that will help ensure progress towards licensure. This will require well-characterized challenge material with appropriate histories in terms of clinical isolation and limited passaging. Deep

sequencing of the working stocks of these strains would provide additional characterization of their quasispecies make-up, but consensus sequences would be required at a minimum.

The animal models of aerosol infection will require further development in order to serve as the animal model to be used in pivotal efficacy studies. The lack of aerosol cases in nature poses a challenge, although there are nearly 150 documented cases of accidental laboratory exposure to VEEV aerosols in the literature.[52] Thorough comparison of the pathological findings in the cynomolgus macaque to those from human cases will be critical. However, there may be opportunities to bridge data from aerosol exposures to subcutaneous VEEV exposures as well. Comparisons could be made between subcutaneous exposures in macaques to natural disease in humans, then between subcutaneous and aerosol exposures in macaques to provide the three-way comparison. That being said, the human cases of aerosol exposure, although limited, could be the key data points that will enable the use of cynomolgus macaques in the Animal Rule.

Identification of correlates of protection will be another critical piece of information for a successful WEVEE vaccine program. It will be essential, given a vaccine candidate, to determine the mechanism by which it confers protection. This would enable measurements of the key immunological parameter(s) in clinical trials that are indicative of protective efficacy and demonstrated in pivotal animal studies. In that light, validated assays to measure these parameter(s) will also be needed.

Multiple experimental vaccines are continuing progress towards a point at which clinical trials are justifiable. Regarding licensure of a vaccine against aerosol exposures, the Phase I and II safety and immunogenicity trials would be conducted in a manner similar to that of products for other infectious diseases. However, a Phase III study would not include a field efficacy endpoint. Instead, Phase I/II clinical testing would provide an expanded immunogenicity and safety study to be accompanied by a pivotal animal study. In the case of the WEVEE vaccine, the pivotal animal study would likely utilize a IAB strain of VEEV given its history in offensive programs, relevance to human clinical disease, and the limited number of aerosol exposures of humans in laboratory accidents. With the goal of a trivalent vaccine in mind, two options could be pursued. The more straightforward option would consist of additional pivotal animal studies showing protective efficacy against a small set of the most relevant strains. Alternatively, and if a correlate of immunity proves to be well-defined for the vaccine in question, material (e.g. serum) from the clinical trials could be utilized in passive transfer/adoptive transfer experiments in the appropriate animal model and/or *in vitro* assays to measure immunological cross-reactivity. In all likelihood, it will be some combination of the above. However, continued emphasis on the biology of encephalitic alphaviruses in terms of their genomic diversity, the structural biology of the envelope proteins, and their comparative virulence will provide the rationale needed to inform well-designed studies that will prove the breadth of vaccine efficacy. Further development of the animal models and assays will enable the eventual licensure of these vaccines. Keeping these issues in mind, a safe and effective vaccine will be delivered to the Warfighter that may also be of use to the public health and agricultural communities.

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Table 1: Snapshot of current WEVEE vaccines in development[a]

Vaccine Platform	Breadth of Protection	Route of Challenge	Route of Vaccine Administration	Animal Species
DNA Vaccine	WEEV, EEEV, and VEEV	Aerosol	Intramuscular[b]	Cynomolgus macaque
Viral Replicon	EEEV, and VEEV[c]	Aerosol	Subcutaneous	Cynomolgus macaque
Virus-like Particle	WEEV, EEEV, and VEEV	Aerosol	Intramuscular	Cynomolgus macaque
Inactivated Viruses[d]	WEEV, EEEV, or VEEV	Aerosol	Various	Balb/c mouse

[a]Only includes four lead vaccines that have been evaluated in terms of trivalent protection. This also only highlights the highest level of results in terms of breadth of protection, route of challenge, and animal species.
[b]Includes electroporation for delivery of the vaccine.
[c]Potential protection against EEEV, but additional experiments would be required to sufficiently address protective efficacy. Additional testing would also be required to thoroughly assess protection against WEEV.
[d]Evaluated protection elicited by each individual species inactivated by formalin, gamma irradiation, and 1,5-iononapthyl azide. This also assessed differences among intranasal, intramuscular, and subcutaneous administration of each inactivated virus. Results varied as a function of method of inactivation, route of administration, and virus species.

Figure 1: Maximum likelihood tree and tanglegram of whole genome verus E1 gene of VEEV genomes.

Maximum likelihood phylogenies were generated from multiple sequence alignment of the genomes. The number of splits shared between different trees was calculated using FastTree, and visualized with tanglegrams. The tanglegram was created in Dendroscope. Equivalent branch rotations which did not change the relationships within a tree are performed by an algorithm to minimize the number of crossing lines between trees. The names of the VEEV strains included serotype, country of origin, year of collection, strain name, and the host species from which it was isolated. Countries included Colombia (CO), Venezuela (VE), Ecuador (EC), Peru (PE), United States (US), Guatemala (GA), Trinidad (TR), Panama (PA), Mexico (ME), Honduras (HO), French Guiana (FG), Belize (BE), Brazil (BR), and unknown (u). Host species included hamster (ham), human (hum), horse (hor), donkey (don), mosquito (mos), and unknown (u).

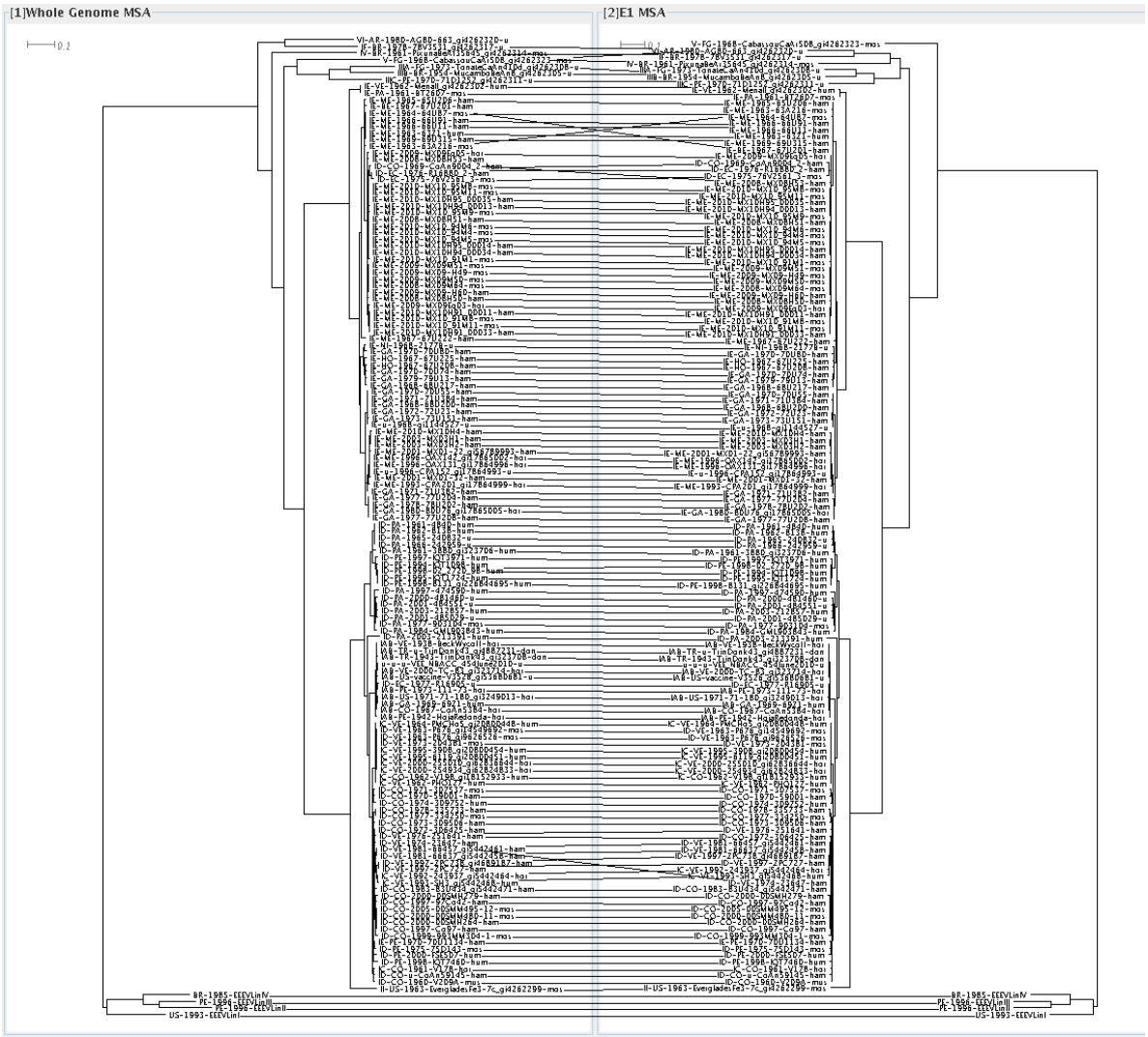

Figure 2: Maximum likelihood tree and tanglegram of E1 versus E2 genes of VEEV genomes.

The trees and the tanglegram were generated as described in Figure 1. The phylogenetic differences between E1 and E2 shows that major genome groupings are the same, and only the relationship among closely related strains within clades differs between the two genes.